Production of triterpenoid compounds from ganoderma lucidum spore powder using ultrasound-assisted extraction

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ABSTRCT

When ingested as a dietary supplement, Ganoderma lucidum spore powders (GLSP) provide various health benefits such as enhanced immunity, liver protection and anti-cancer effects. In this study, triterpenoid extraction from GLSP was achieved using an ultrasound-assisted process which was optimized using response surface methodology (RSM). Ultrasound-assisted extraction (UAE) was also compared to the most conventional chemical extraction method. For UAE, optimum extraction conditions were found to be ethanol concentration=95 %v/v; solvent to solid ratio=50:1 mL/g; ultrasound time=5.4 min; ultrasound power=564.7 w and ultrasound probe distance=8.2 cm. At optimal UAE conditions, no significant differences were found between experimental (0.97 ± 0.04 %) and predicted values (0.99 %); which indicates appreciable correlation at the 97 % confidence interval. The findings show the application of Box-Behnken design (BBD) to predict and optimize triterpenoid yield for UAE of triterpenoid from GLSP. Furthermore, glucose consumption was 2.68 times that of control samples when tested with insulin-resistant HepG2 cell, showing potential use in type 2 diabetes. In addition, triterpenoid extracts show good biocompatibility and inhibition of antioxidant activity.

Keywords: *Ganoderma lucidum; chemical extraction; ultrasound extraction; food function; bioactivities.*

1. Introduction

Ganoderma lucidum is a fungus from the Ganodermataceae family. *G. lucidum* is a traditional Asian medication typically used as functional foods to promote health and longevity, especially in China [1]. By cultivating various components from this fungi, an array of products have been commercialized globally as dietary supplements [2]. The fruiting body, mycelia and spores of *G. lucidum* have traditionally been used for preventing and treating various medical conditions including, but not limited to, hepatitis [3], hypertension [4], nephritis [5], bronchitis [5] and some cancers [6]. The chemical composition of *G. lucidum* is very complicated; containing 11 active substances including polysaccharides, organic germanium, triterpenoids, oils, inorganic ions and sterols [7].

Ganoderma lucidum spore powder (GLSP) is a spore of *G. lucidum.* These spores are small oval germ cells that have been commercialized used as dietary supplements [8]. These cells house all the genetic material of *G. lucidum* [9] and hence all the health effects [10, 11]. Each spore is a living organism of 4-6 μm size. It has a double-walled structure surrounded by hard chitin cellulose. It is this sheath of cellulose that makes limits the absorption of the spores in the human body. Disrupting this wall enables direct spore absorption in the human stomach [12]. Isolated or extracted triterpenoids from *G. lucidum* have been reported to be responsible for the pharmaceutical activity of the fungi [13]. To date, over 400 triterpenoids have been found, with many more still being discovered [14, 15]. Triterpenoids compose of three terpene or six isoprene units and are characterized into 6 broad categories based on the number of rings present: acrylic, monocyclic, bicyclic, tricyclic, tetracyclic, pentacyclic or hexcyclic triterpenoids. These compounds have shown broad biological activity including anticancer, anti-inflammatory [16, 17], antioxidant and anti-proliferation capabilities. Triterpenoids have also found to possess anti-angiogenesis and anti-lymphatics abilities [18]. Whilst there are many examples on the successful utilization of triterpenoids, there are very few studies which show the potential of GLSP. Hence, it is necessary to explore

the constituents and bio-activity of triterpenoids present in GLSP, which helps to optimize and develop triterpenoid products. In this study, antimicrobial and antioxidant properties of GLSP triterpenoids was evaluated. Furthermore, the effect of triterpenoids on glucose consumption in normal and insulin-resistant HepG2 cell lines was assessed.

The step first in utilizing these bioactives is their extraction from GLSP. More traditional methods have some limitations. The major challenges are long extraction times and the amount of solvent required. Some methods also have low efficiency [19]. However, due to material development and evolution in equipment, more efficient techniques have been developed which have found to efficiently isolate and extract the triterpenoids from plant materials. Ultrasonic Assisted Extraction (UAE) has many advantages over more conventional methods including but not limited to shorter processing time, ambient pressure and temperature operation, reduction in solvent use, greater extraction rate, economical process and increased product purity [20]. In addition to these, wide applicability, easy maintenance and maintenance of equipment area are further added benefits [21, 22]. In many cases, the whole procedure is simpler as it involves fewer operations (steps) and is thus less prone to contamination. However, the shortcomings of UAE are that the effective action area (ultrasonic) is restricted by the ultrasonic attenuation factor. If the diameter of the extraction tank is too large, an ultrasonic blank area is formed on the peripheral wall of the tank [23]. In addition, controlling factors such as solvent to material ratio and working distance can alter the extraction efficiency.

Ultrasonic Assisted Extraction (UAE) is based on the principle of acoustic cavitation force, as the main driving force, which is capable of generating continuous compression and rarefaction in the presence of an extraction solvent. This results in the formation of microbubbles with internal pressure giving rise to "micro-explosions." These produce microscopic yet significant shock waves which allow subsequent release of bioactive compounds from plant materials [20, 24]. Several mechanisms involved in UAE have been identified. One of these mechanisms is erosion upon

ultrasound exposure, which improves the accessibility of the solvent by imploding bubbles on the surface of the plant material. In addition, particle collisions under ultrasonic waves (which cause a reduction in the particle size) can facilitate mass transfer [25]. Sonoporation and the sheer stress mechanism produces the collapse of bubbles in the solvent and is able to improve the penetration of liquid and alter the permeability of the cell membranes [26]. However, many process parameters affect extraction efficiency, such as solvent type, solvent to solid ratio, ultrasonic duration and temperature. [27]. To promote the potential and application of GLSP, it is critical to identify the optimal parameters and response surface methodology (RSM) is an effective tool to do so [28]. Using this mathematical technique, triterpenoid extraction efficiency can be greatly improved.

The research presented here uses ultrasound treatment to assist the extraction of triterpenoids from GLSP. The effects of four key extraction parameters using solvent reaction were investigated: extraction temperature, ethanol concentration, extraction duration and ratio of solvent to material. Furthermore, the effects of three ultrasound-assisted extraction parameters (ultrasound power, ultrasound time and ultrasound distance) on the yield of triterpenoids was assessed and used for process optimization using RSM with a five-level, three-variable Box-Behnken design (BBD).

2. Materials and Methods

2.1 Materials

Ethanol and glacial acetic acid were purchased from Taicang Shanghai Test Reagent co. (Jiangsu, China); Perchloric acid was purchased from Shanghai Wokai Biotechnology Co. (Shanghai, China); Oleanolic acid was purchased from Adamas-beta (Shanghai, China); Vanillic was purchased from Shanghai Aladdin Bio-Chem Technology Co., LTD (Shanghai, China). All the reagents used were of analytical grade. Dried GLSP was obtained from Tianhe Agricultural Group (Zhejiang, China) and was stored in sealed (air tight) flasks at −4°C in complete darkness until experimentation. The production address is 514 West Street, Longquan City. We purchased the finished bagged products and the hygiene indicators meet the requirements of GB7096. The product was used as obtained without further purification.

2.2 Extraction methods

In chemical extraction, ethanol was selected to investigate the effect of controlling parameters on the extraction of triterpenoid from GLSP. Firstly, the effect of temperature on triterpenoids extraction was investigated. Dried GLSP (1 g) was introduced to a standard 50 mL centrifuge tube with 95% v/v ethanol added at a liquid-to-solid ratio of 20:1 (ethanol volume: *GLSP* quantity). Extraction was performed for 1 h at different extraction temperatures (0 ~ 90°C). The centrifugation conditions during experiments were as follows: speed of 6000 rpm at a temperature of 20°C for 5 min. Secondly, the influence of ethanol concentration on the yield of triterpenoids was analyzed. At 60°C, 20 mL of different ethanol concentrations (60 ~ 95% v/v) were added and the extraction process was performed for 1 h. Thirdly, the extraction duration was investigated between the range of 15 and 90 min at 60°C using 20 mL of 95% v/v ethanol. Finally, the optimal liquid-to-solid ratio was studied in a range from 20 to 60 the concentration of ethanol was 95% v/v and the extraction was performed for 1 h at 60°C.

In ultrasound-assistant extraction, the high intensity ultrasound irradiation was applied to GLSP suspended in 50 mL of ethanol solution using an ultrasonic generator (GBP-USC401A, Hangzhou Guobiao Ultrasonic Instrument Co., Zhejiang, China). Ultrasound power output was managed according to details provided from the equipment provider. The output ultrasonic power can be regulated from 250 to 1000 W. The water container (200 × 150 × 150 mm) was filled with 3 L of water. An iron support stand was used to maintain the distance between ultrasound probe and sample at predetermined specific distances. Three factors were chosen to assess their effect on the recovery of triterpenoids from GLSP through single-factor experiments: ultrasound power, ultrasound time, and ultrasound distance (distance between ultrasound probe and centrifuge tube). When one variable was assessed, the other variables were kept constant. The constant values of ethanol concentration and liquid-to-solid were 95% v/v and 50:1 respectively.

2.3 Box-Behnken Design (BBD)

Box-Behnken Design was used to determine the optimal conditions of UAE. The study of the effects of all defined independent variables was performed using a one-factor procedure. This was to determine the initial range of processing variables. The selected three main independent variables, ultrasound power (A, W), ultrasound time (B, min) and ultrasound distance (C, cm). Table 1 shows their associated codes and

levels. The experiment has 12 factorial experiments and three replicates of the central point (Table 2). The yield of triterpenoids was used as the dependent variable of the independent variables given in Table 2.

The response surface models were fitted by means of least-squares calculation using the following second-order polynomial equation:

$$Y= a_0 + b_1A + b_2B + b_3C + c_{12}AB + c_{13}AC + c_{23}BC + d_1A^2 + d_2B^2 + d_3C^2 \quad (1)$$

Where Y is the predicted response; a_0 is the interception; b_1, b_2 and b_3 are the linear coefficients of ultrasound power (A), ultrasound time (B) and ultrasound distance (C), respectively; c_{12}, c_{13}, and c_{23} are the interaction coefficient of ultrasound power, ultrasound time and ultrasound distance, respectively; d_1, d_2 and d_3 are the squared coefficient of ultrasound power, ultrasound time and ultrasound distance, respectively. Statistical analysis was performed using the software Design Expert (Version 8.0.6, Stat-Ease Inc., Minneapolis, MN, USA).

2.4 Quantification Analysis

The determination of the total content of triterpenoid saponins was performed according to previous work [29]. The standard curve, which was used as the benchmark for the yield determination was obtained as follows. 20 mg oleanolic acid was added to a volumetric flask and was made up to 100 mL with absolute ethanol (0.2 g * L-1). The different volumes of oleanolic acid (0, 0.2, 0.4, 0.6, 0.8 and 1.0 mL) were transferred into a 10 mL test tube, respectively. The solvent was heated to evaporation (100°C) in an electric thermal constant temperature tank (DK-8D, Shanghai, Jinghong Experimental Equipment Co., Shanghai, China). To this 0.4 mL of a 5% w/v vanillin-acetic acid solution and 0.6 mL perchloric acid were added and incubated at 60°C for 15 min. The tubes were removed from the heat and cooled in an ice bath for 3 min. The volume in each tube was made up to 5 mL with glacial acetic acid. The tubes were subsequently kept at room temperature (25°C) for 15 min. The samples were scanned with a blank solution as a reference within the range of 200-700 nm using a UV spectrophotometer (SHIMADZU, Japan). The maximum absorption (A) of oleanolic acid at 545 nm was determined using a 1 cm glass cell. The extracted compound was dissolved in ethanol to prepare a pre-determined concentration. The triterpenoids content was determined using UV spectroscopy as described above. The contents of triterpenoid in GLSP were determined using the standard curve.

2.5 HPLC-Q-TOF-MS analysis

In order to identify the compounds being extracted using ultrasound assisted method, we used a Fast High Performance Liquid Phase-Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometer (AB Sciex Triple TOF 5600+, AB Sciex, USA). The mass range (m/z) of the instrument was 50-10000 m/z, and the resolution was greater than 40000.

2.6 Antioxidant Activity

Radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) was performed in below. Firstly, 0.2 mM DPPH and samples of different concentrations from 0 to 400 μg/mL were prepared. 100 μL of DPPH was then added to a 100 μL sample in a 96-well plate. The control group replaced the DPPH with ethanol, and the blank group replaced the sample with distilled water. After 30 min at room temperature, the absorbance at 517 nm was measured with a microplate reader. Each experiment was repeated 5 times. For comparison, commercial synthetic antioxidants (ascorbic acid) were tested in the same manner. The results were expressed as a percentage reduction absorbance shown by the sample with respect to the DPPH solution. The formula was as follows:

DPPH scavenging (%) = [(control absorbance – sample absorbance) / control absorbance] × 100. (2)

Radical scavenging activity using ABTS radical scavenging activity in this study was based on a slightly modified method deployed [30]. Total Antioxidant Capability Test Kit (ABTS Rapid Method) (Beyotime Biotechnology Co., Shanghai, China) was used for the test. Firstly, 20 μL of peroxidase working solution was added to each well of a 96-well plate. Then, 10 μL of ethanol solution was added to the blank control well, and 10 μL of selected varying concentrations of the sample were added to the sample detection well. Subsequently, 170 μL of ABTS working solution was added to each well and mixed. The absorbance was measured at 414 nm after 6 min incubation period at room temperature (25°C). The calculation formula for ABTS scavenging activity is as follows:

ABTS scavenging (%) = [(control absorbance – sample absorbance) / control absorbance] × 100. (3)

2.7 Cell viability analysis

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Corning, USA) and were supplemented with 10% Fetal Bovine Serum (FBS, Gibco)

and 1% antibiotic (Penicillin 100 U/mL, Streptomycin 100 μg/mL). Cells were cultured in an incubator in 5% CO_2 at 37°C. The effect of triterpenoids on HepG2 cell viability was investigated using a CCK-8 assay. Firstly, varying quantities of the triterpenoid extract were added to the DMEM medium at concentrations of 0.015, 0.03, 0.06, 0.1 and 0.2 mg/mL. HepG2 cells (grown in log phase) (100 μL) were introduced into 96-well plates and cultured for 24 h to allow cell adherence. The original medium was removed and the medium containing the triterpenoid extract was replaced and cultured for 24 h. The medium was mixed with CCK-8 at a ratio of 10:1. After 24 h, the previous medium was removed and 100 μL of the mixture was added to each well and the plate was cultured in an incubator (37°C). The absorbance was measured using a microplate reader at pre-determined time points. The cell survival rate was calculated as follows:

Cell survival rate = [(As – Ab) / Ac – Ab)] × 100% (4)

As: Experimental well (cell-containing medium, CCK-8, triterpenoid extract)

Ac: Control well (cell-containing medium, CCK-8, no triterpenoid extract)

Ab: Blank well (a medium free of cells and triterpenoid extract, CCK-8)

2.8 Glucose consumption assay

A glucose assay kit (Glucose oxidase-peroxidase method, Shanghai Rongsheng Biological Pharmaceutical Co., Ltd, China) was used to determine glucose consumption based on a previous study [31]. HepG2 cells (log phase growth) were added into 96-well plates and cultured for 24 hours. Following cell attachment, triterpenoid-containing medium was added and allowed to incubate for 24 hours. The cell culture samples were then rinsed with triterpenoid-free medium, and replace the triterpenoid-free medium for further 12 hours. Cell imaging was conducted using optical microscopy. The cell culture medium was aspirated and the supernatant was subsequently mixed with a reagent liquid (Mixture of R_1 and R_2 in the glucose assay kit), and allowed to stand at 37°C for 15 min. Thereafter, liquid absorbance was measured at 505 nm using a microplate reader (spectra Max 190, NanoDrop, USA). The glucose concentration in wells hosting cells was subtracted from glucose concentration in blank wells to obtain glucose consumption quantity, as shown in Eq. 5:

Glucose (mmol/L) = sample absorbance / calibration absorbance × calibrator concentration. (5)

An insulin-resistant cell model was induced and used according to a previous study [32]. HepG2 cells (log phase growth) were added into a 96-well plate and cultured for 24 hours. Medium containing insulin was replaced for 36 hours. Then the triterpenoid-containing medium was replaced for 24 hours. The following steps were identical to the procedure in 2.8.1.

2.9 Statistical analysis

All experiments were performed in triplicate. The results were expressed as mean value ± standard deviation. The statistical analysis was carried out using Origin Pro 2017 and WPS.

3. Results and discussion

3.1 Calibration and determination of triterpenoid content

A standard curve (for triterpenoid saponins) was obtained using UV analysis at a wavelength of 545 nm. It can be seen from the figure that different quality oleanolic acids have maximum absorption peak at 545 nm. This can be seen as a characteristic peak. A linear correlation relationship absorbance and concertation is demonstrated using a regression model:

$$A = 6.22C - 0.0298, (R^2 = 0.998) \quad (6)$$

Where C (mg/mL) is the concentration of triterpenoid. A is the absorbance at 545 nm. Equation (7) was used to calculate yield (Y) based on the following equation:

$$Y = (A + 0.0298) / 6.22 \times v / m \times 100\ \%\ (\%;\ w/w) \quad (7)$$

Where V is the total volume of extraction solvent (mL), and m is the mass of ginseng sample (g).

3.2 Optimization of chemical (ethanol) extraction

Chemical based extraction of triterpenoid from GLSP was investigated focusing on four factors (extraction time, extraction temperature, ethanol concentration and liquid-to-solid ratio).

3.2.1 Effect of extraction temperature

The effect of temperature on the extraction rate of triterpenoids was studied. Extraction was performed using 20 mL ethanol (95% v/v) for 1 h at six different temperatures (0, 50, 60, 70, 80 and 90°C). As shown in **Figure.1a**, the yield increases with increasing temperature until 60°C, after which a slight reduction is observed. Due to the increased desorption of analytes from the active sites in the matrix, increased process temperatures result in higher extraction efficiencies. In addition, the dissolution

rate of the analyte is expected at higher temperatures. Both surface tension and solvent viscosity will decrease with increasing temperature, which is known to improve sample wetting and matrix permeability, respectively [33]. When a process temperature above 60°C is used, a slight decrease in yield is most likely due to the diffusion of the enhanced solvent to the interior region of the matrix. Furthermore, as triterpenoid saponins extraction rate increases, an increase in matrix component co-segregation has been demonstrated previously [34]. For this reason, 60°C was selected as the optimal temperature for ethanol extraction.

3.2.2 Effect of ethanol concentration

Most natural antioxidants from plants are readily soluble in low polarity organic solvents. Water-based co-solvent systems using methanol, ethanol and acetone have been previously used for natural product dissolution [35, 36]. In this study, ethanol concentrations ranging from 60 to 95% v/v were selected to investigate the impact of solvent concentration on triterpenoid yield. For this, other parametric extraction conditions remained constant (solvent to material ratio = 20 mL/g, extraction time = 1 h and process temperature = 60°C). As shown in **Figure.1b**, triterpenoid yield increases from 0.16 ± 0.003 to 0.71 ± 0.082% when ethanol concentration is increased from 60 to 95% v/v. Since triterpenoid possesses polar groups the solubility is expected to increase and has been shown previously for other plant components [37]. A maximum triterpenoid yield is achieved when using an ethanol concentration of 95% v/v and this value was selected for subsequent experiments.

3.2.3 Effect of extraction time

Process (extraction) time is a vital factor which impacts triterpenoid yield; whilst also impacting economic factors. Extraction time is identified as the period of contact between solvent and solid base matrix. Prolonged extraction time at elevated temperatures have been shown to accelerate molecular movement and also alter electrical conductivities of extraction solvent and plant [38]. To investigate the impact of extraction time, triterpenoid was extracted using ethanol (20 mL, 95% v/v) at 60ºC. A series of extraction times were selected between 15 to 90 min. As shown in **Figure.1c**, the results indicate triterpenoid yield increases with extraction time up until 60 min; after which a reduction in yield is observed. The greatest yield (0.68%) was observed at 60 min. Extraction times longer than 60 min leads to triterpenoid decomposition due to prolonged contact at optimal temperature (60ºC). This phenomena has also been

observed for wheat straw extraction using steam explosion and ethanol extraction [39]. Therefore, an extraction time of 60 min was selected as the optimum.

3.2.4 Effect of ratio of liquid-solid ratio

Determining the optimal liquid-solid ratio for the extraction process is crucial as it directly imparts uniform heat on the selected material. Extremely low liquid-solid ratios lead to super-saturation of solute in solvent, which impedes mass transfer and speed [40]. Increasing the liquid to solid ratio generally increases the solvent volume on the (inner and outer) outer regions of the plant component. This enhances mass transfer kinetics and contact between the substrate and the solvent. However, very high liquid-solid ratios lead to an over-cell-wall diffusion distance for solute, thus interfering with its dissolution rate [41]. Triterpenoid was extracted with ethanol (95% v/v) for 1 h at 60ºC, and the ratio of solvent to material varied from 20:1 to 50:1 mL/g. As seen in **Figure.1d**, triterpenoid yield was enhanced (from 0.68 ± 0.031% to 1.02 ± 0.030%) upon increasing solvent to material ratio. When the solvent to material ratio is > 50:1 mL/g, no further change in triterpenoid was observed. In general, an increase in solvent (by ratio) increases contact due to improved solubility (triterpenoid component). However, at a liquid to solid ratio of 50:1 (mL/g) an equilibrium is reached and any increase in ratio will not impact extraction yield. Thus, 50:1 mL/g was considered as the optimal ratio of solvent to material for the extraction process.

3.3 Optimization of ultrasound-assisted extraction

Ultrasound power, ultrasound time and ultrasound distance were all explored for their impact on triterpenoid yield. Temperature impacts the UAE and also changes during the process. The temperature can be varied as a controlling factor during extraction [42]. We studied the relationship between temperature and irradiation time. The results are shown in **Figure. 2**. In this experiment, ultrasonic time was varied in the range 0-30 min. Here, the temperature increased by 18°C; from 12 °C at 0 min to 32°C at 30 min. Therefore, water temperature was monitored and recorded constantly and water was replaced with a fresh supply to ensure experimental temperature was kept below 35°C.

3.3.1 Effect of ultrasound power

The level of ultrasound power deployed is directly linked to cavitation ability [43]. Movement of water molecules is accelerated upon application of ultrasound; which subsequently leads to rapid bubble formation and collapse. These events occur at the plant surface interface and hence generate heat and pressure [44]. Temporary acute

impact waves and high-speed efflux are known to disrupt the cell wall [45], releasing components within cells. The effect of ultrasound power (from 450 to 750 w) on extraction efficiency was investigated. Triterpenoid was extracted from 1 g GLSP with control conditions of 50 mL ethanol (95% v/v) and a process time of 10 min. As shown in **Figure.3a**, triterpenoid yield decreases slightly when the ultrasound power is increased from 450 to 750 W, with the greatest yield (1.16%) obtained at 450 W. The local heating effect arising from sonication has been shown to induce thermal degradation, leading to a reduction in extraction efficiency [34]. For this reason, 450 W was set as the optimum ultrasound power.

3.3.2 Effect of ultrasound time

The impact of ultrasound application time was explored with the upper and lower test limits set to 0.5 to 30 min. For this 1 g GLSP powder was used and base conditions were set as 50 mL ethanol (95% v/v) and ultrasound power = 450 w. As shown in **Figure.3b**, increasing ultrasound application time from 0.5 to 5 min leads to greater triterpenoid yield (from 1.00 ± 0.017% to 1.19 ± 0.08%). An application time beyond 5 min leads to a reduction in yield, which indicates triterpenoid degradation. This has also been observed for the extraction of phenolic compounds [46]. Therefore, an ultrasound time of 5 min was chosen as the optimum.

3.3.3 Effect of ultrasound distance

The distance between the probe tip and container affects energy transmission to the sample, which in turn impacts extraction efficiency [47]. An ultrasound distance from 1 to 8 cm was investigated. For this, triterpenoid was extracted from 1 g GLSP using the following conditions; ultrasound exposure (450 w), ethanol 50 mL (95% v/v) and an ultrasound application time of 10 min. As shown in **Figure.3c**, triterpenoid yield increases with increasing ultrasound distance, although a reduction in extraction is observed at distances of equal to or more than 11.5 cm. When the distance is less than 8 cm, triterpenoid may undergo degradation due to various types of energy being generated and close proximity (e.g. heat and vibration). At 11.5 cm and above, ultrasonic energy is weaker. Therefore, a probe tip to container distance of 8 cm was deemed optimal.

3.4 Response surface methodology (RSM)

RSM was used for optimizing the extraction process, and all test equipment was calibrated and experimental procedures were kept accurate. All experiments were performed by exactly the same personnel and ambient environments were near identical.

3.4.1 Model Fitting

The Box-Behnken (BB) design model was used to study interactions among variables A, B and C and to determine optimal levels. The results are represented in **Table 2**. By fitting a second-order polynomial model of Equation (1) to the obtained responses, parametric values are obtained. Therefore, the resulting model for UAE is:

$$Y=1.06 - 0.10A - 3.750 \times 10^{-3}B + 0.019C + 2.500 \times 10^{-3}AB + 2.500 \times 10^{-3}A - 5.000 \times 10^{-3}BC - 0.048A^2 - 0.081B^2 - 0.14C^2 \quad (8)$$

The test results were analyzed using ANOVA and multiple linear regression. The significance of regression equation was evaluated by values of F and p. **Table 3** details the regression model of triterpenoid yield. The analysis shows that the model was significant at an F-value of 79.84 ($p < 0.0001$). The model's adaptability was studied by lack of fit index, and the lack of fit term was not significant ($p > 0.05$), indicating the model predicted the variation of the tests conducted successfully.

Table 4 shows model-fitting results, expressed as coefficient (R^2), modified coefficient of association (R_{Adj}^2), predicted modified coefficient of association (R_{Pred}^2) and varied coefficient (CV). The association coefficient of the model $R^2 > 0.99$, indicates that the test value is highly correlated with the predicted value. **Table 4** shows that for this model, R_{Adj}^2 was slightly smaller than R^2, showing the discrepancy between R_{Pred}^2 and $R_{Adj}^2 \leqslant 0.12$; indicating a reasonable range of fluctuation [48]. Herein, Adeq Precision in the model was more than 24, which validates fitting of the extraction process. These results show the model to adequately represent the real relationship between response and independent variables.

3.4.2 Response Surface Analysis

To determine interactive effects of independent variables on triterpenoid extraction yield, three dimensional response surface and contour plots were obtained and are displayed in **Figure.4**. The plots were generated by plotting responses using the z-axis against two independent variables, while keeping other independent variables constant. **Figure.4(a) and (d)** show three-dimensional and two-dimensional response surface plots for interactive effects of ultrasound power and ultrasound time on response values at fixed ultrasound distance. An increase in ultrasound time from 1 to 5 min improves triterpenoid yield, while the yield decreases with an increase in ultrasound power from 450 to 600 w. **Figure.4(b) and (e)** show three-dimensional and two-dimensional response surface plots for interactive effects of ultrasound power and

ultrasound distance on response values at a fixed ratio for ultrasound time. Triterpenoid yield is shown to increase with increasing ultrasound distance and reaches a peak value at 8 cm, while an extension of ultrasound power decreases the yield. **Figure.4(c) and (f)** show three-dimensional and two-dimensional response surface plots for interactive effects of ultrasound time and ultrasound distance on response values for fixed ultrasound power. Increasing either ultrasound time or ultrasound distance leads to enhanced triterpenoid yield. This reaches a peak value when the ultrasound time is 5 min and the ultrasound distance is 8 cm. The results indicate that ultrasound power is the major factor affecting the responses at a significance level of $p < 0.01$.

3.4.3 The optimization of parameters

A variety of factors could be assessed using multiple-responses in order to select the suitable craft parameter for the actual process [49]. The optimum conditions for ultrasonic-assisted extraction of triterpenoids by regression model are as follows: ultrasound power 564.74 w, ultrasound time 5.42 min, ultrasound distance 8.25 cm, and the yield of triterpenoids under such conditions was theoretically predicted as 0.99%. The actual operating conditions were considered in order to modify the optimal conditions as follows: ultrasound power 525 w, ultrasound time 5 min, ultrasound distance 8 cm. To ensure the accuracy of the prediction, it was necessary to repeat the tests under the same optimization. The mean yield of triterpenoid was 0.97 ± 0.04%, which was not significantly different from the predicted theoretical value. In summary, the RSM can fairly reflect actual results of triterpenoids in *G. lucidum.*

3.5 Effect of UAE treatments on micro-structure of plant materials

The heat assisted ethanol extraction condition of triterpenoids from GLSP was optimized by using the response surface methodology (RSM) as reported [50]. However, the main disadvantage of this process was the time taken to complete extraction which led to a decrease in triterpenoids compounds productivity. In order to overcome the problem, ultrasound assisted extraction technology was used. In addition, its mechanism of extracting triterpenoids was observed under scanning electron microscope. There was no significant difference between the micro-structures of plant materials obtained from the non-extracted powder (**Figure. 5a**) and samples treated with heating extraction (**Figure. 5b**). In contrast, the UAE caused evident structural changes in the surface by destructing the plant tissues of GLSP (**Figure. 5c**). This revealed that the high temperature had little effect on GLSP, and due to the effects of

ultrasound which cracking and deformation of the spores. In the meanwhile, it was maybe the reason that the increasing yield of triterpenoids from GLSP.

3.6 Triterpenoid profiles by HPLC-Q-TOF-MS

Figure. 6 shows representative HPLC-Q-TOF-MS results for the obtained triterpenoid extract. The triterpenoid extract contains 9 major compounds. As shown in Figure. 6 (a), there was ganoderic acid I (peak 1), lucidenic acid D (peak 2), ganoderenic acid D (peak 3), ganodernoid C (peak 4), ganoderic acid DM (peak 5) and ganodermic acid TQ (peak 6). In Figure. 6 (b), ganoderenic acid D (peak 1), ganoderiol I (peak 2) and ganoderic acid C2 (peak 3) can be observed.

3.7 The DPPH radical-scavenging activity

DPPH is a well-established method to evaluate free radical scavenging activity of natural compounds [51, 52]. The radical scavenging activity of triterpenoid extract was evaluated by comparing the DPPH scavenging capacity of different concentrations of triterpenoids **(Figure. 7**). From the figure, it can be seen that as concentration increases, the radical scavenging activities also increases. When the concentration of triterpenoids was 400 μg/mL, the percentage inhibition of DPPH radical was 62.16%. In this study, free radical compounds show maximum absorption at 517 nm and are readily removed by antioxidant action [51, 53]. DPPH removal assay is based on DPHH reduction in the presence of a proton donor [51]. In this study, as the concentration of triterpenoids is increased, the scavenging ability is also higher based on enhanced proton concentration. In addition, earlier studies indicate protein content is an important factor, and is known to promote DPPH radical scavenging activity of biomolecules [54]. According to our results, the triterpenoid extract exhibited mild `antioxidant activity, but was more effective than crude extract of well-known plants (e.g. Platycodon grandiflorum) [55]. This is attributed to the intrinsic nature of the extract triterpenoids from GLSP.

3.8 The ABTS radical-scavenging activity

ABTS assay is used to evaluate a compounds (plant based) antioxidant capability [56]. Scavenging hydrogen radicals is an important antioxidant characteristic. ABTS•+, a protonated free radical with a maximum characteristic absorption at 414 nm, decreases with removal of hydrogen radicals [57]. The radical scavenging activity of triterpenoid extract was evaluated by comparing the ABTS scavenging capacity at various triterpenoids concentrations (**Figure. 8**). As shown in **Figure. 8**, ABTS scavenging activity of triterpenoids extract correlated positively with its concentration in medium. When the concentration of triterpenoids was 400 μg/mL, the percentage

inhibition of ABTS radical was 65.64%. In this study, as the concentration of triterpenoids is increased, the inhibition of ABTS•+ production was enhanced, indicating an increase in antioxidant capacity [57]. The experimental results were similar to those of DPPH, which also demonstrated that triterpenoids had moderate radical scavenging activity. Scavenging ability correlates with previous studies and elucidates the potential role of triterpenoids as an antioxidant.

3.9 Cell viability assays

The effect of triterpenoids concentration on HepG2 cell survival rate was investigated. As sown in **Figure. 9**, concentrations ranging from 0.015 to 0.2 mg/mL were studied. Increasing the Triterpenoids concentration from 0.015 to 0.2 mg/mL, resulted in a positive correlation with HepG2 cell viability. When the concentration of triterpenoids was 0.015 mg/mL, the cell viability was 110.98% and at a concentration of 0.2 mg/mL, the cell viability was 120.91%. In this regard, triterpenoids demonstrate good HepG2 cell biocompatibility over the concentration range deployed in this study.

3.10 Effect of triterpenoid extract on HepG2 cell glucose consumption

The effect of triterpenoid extract on HepG2 cell glucose consumption is shown in **Figure. 10**. As shown in **Figure. 10a**, at triterpenoid extract concentrations of 0.015 or 0.03 mg/mL, no significant difference in glucose consumption between metformin and triterpenoid extract treated samples is observed. However, when the extract concentration is 0.06 mg/mL, a significant difference is seen. Metformin is a biguanide that lowers hyperinsulinemia and improves hepatic insulin resistance. Cell morphology is shown in **Figure. 11A** (0.06 mg/mL) and glucose consumption using normal HepG2 cell lines is significantly improved by triterpenoid extract.

Insulin resistant glucose uptake is a prominent feature of type I and II DM (Diabetes mellitus) in experimental models of diabetes [58]. Therefore, glucose consumption in insulin-resistant HepG2 cell lines was assessed. In **Figure. 10b**, metformin significantly increases glucose consumption. When triterpenoid extract concentrations were 0.03 and 0.06 mg/mL, notable glucose consumption values (activity) of 1.80±0.12 and 2.21±0.29 mmol/L, respectively, is observed. Furthermore, the effect of triterpenoid extract on cell morphology was shown in **Figure. 11B**. It can be seen from the figure that the different concentrations of triterpenoids used in the experiment were not toxic to cells. These results indicate triterpenoid extract can modulate insulin sensitivity; making them potential therapeutic agents for the treatment of diabetes.

4. Conclusion

In summary, ultrasound-assisted extraction was successfully used and optimized to obtain triterpenoid from GLSP using RSM coupled with the Box-Behnken design. When compared to conventional chemical extraction (using ethanol alone), the yield is enhanced when using UAE with reduced extraction time. Based on the model, optimum extraction conditions were as follows: ethanol concentration = 95% v/v; ratio of solvent: solid = 50:1 mL/g; ultrasound time = 5.42 min; ultrasound power = 564.74 w and ultrasound distance = 8.25 cm. At optimal UAE conditions, triterpenoid yield was 0.99% and no significant difference between predicted (0.99%) and experimental values (0.97 ± 0.04%) was found. In addition, SEM indicates an increase in triterpenoid extraction rate when using ultrasound may be due to the disruption of matrix structure. Overall, the findings demonstrate that UAE is a more efficient method for extracting triterpenoid from GLSP. In addition, triterpenoid extract with good biocompatibility showed potential use for type 2 diabetes, mild DPPH radical scavenging activity, and inhibition of antioxidant activity.

Acknowledgements

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Reference

[1] Duvnjak D, Pantić M, Pavlović V, et al. Advances in batch culture fermented Coriolus versicolor medicinal mushroom for the production of antibacterial compounds. *Innovative Food Science & Emerging Technologies*. **2016**, 34, 1-8.

[2] Peng L, Yan-Ping D, Xiao-Xia W, et al. Triterpenoids from Ganoderma lucidum and their cytotoxic activities. *Natural Product Research*. **2013**, 27(1), 17-22.

[3] Chen X, Zhang X, Ma Y, et al. Iridal-type triterpenoids with anti-HBV activity from Iris confusa. *Fitoterapia*. **2018**, 129, 126-132.

[4] Rodriguez-Rodriguez R. Oleanolic Acid and Related Triterpenoids from Olives on Vascular Function: Molecular Mechanisms and Therapeutic Perspectives. *Current Medicinal Chemistry*. **2015**, 22(11), 1414-1425.

[5] Eom HJ, Kang HR, Kim HK, et al. Bioactivity-guided isolation of antioxidant triterpenoids from Betula platyphylla var. japonica bark. *Bioorganic Chemistry*. **2016**, 66, 97-101.

[6] Eom HJ, Kang HR, Choi SU, et al. Cytotoxic Triterpenoids from the Barks of Betula platyphylla var. japonica. *Chemistry & Biodiversity*. **2017**, 14(4), e1600400.

[7] El-Mekkawy S, Meselhy MR, Nakamura N, et al. Anti-HIV-1 and anti-HIV-1-protease substances from Ganoderma lucidum. *Phytochemistry*. **1998**, 49(6), 1651.

[8] Fan L, Wang Y, Zhao M, et al. Magnetic Ganoderma lucidum spore microspheres: A novel material to immobilize CotA multicopper oxidase for dye decolorization. *Journal of Hazardous Materials*. **2016**, 313, 122-129.

[9] Ge F-H, Duan M-H, Li J, et al. Ganoderin A, a novel 9,11-secosterol from Ganoderma lucidum spores oil. *Journal of Asian Natural Products Research*. **2017**, 19(12), 1252-1257.

[10] Zhu LF, Li JS, Mai J, et al. Ultrasound-assisted synthesis of chitosan from fungal precursors for biomedical applications. *Chemical Engineering Journal*. **2019**, 357, 498-507.

[11] Zhu L-F, Yao Z-C, Ahmad Z, et al. Synthesis and Evaluation of Herbal Chitosan from Ganoderma Lucidum Spore Powder for Biomedical Applications. *Scientific Reports*. **2018**, 8(1), 14608.

[12] Zhao D, Chang M-W, Li J-S, et al. Investigation of Ice-Assisted Sonication on the Microstructure and Chemical Quality of Ganoderma lucidum Spores. *Journal of Food Science*. **2014**, 79(11), E2253-E2265.

[13] Ruan W, Popovich DG. Evidence of bioactivity from Ganoderma lucidum triterpenoids in cultured cell models. 2012.

[14] Kim HW, Kim BK. Biomedicinal Triterpenoids of Ganoderma lucidum (Curt.: Fr.) P. Karst. (Aphyllophoromycetideae). *International Journal of Medicinal Mushrooms*. **1999**, 1(2), 121-138.

[15] Wang JL, Li YB, Liu RM, et al. A new ganoderic acid from Ganoderma lucidum mycelia. *Journal of Asian Natural Products Research*. **2010**, 12(8), 727-730.

[16] Zhu L-F, Zheng Y, Fan J, et al. A novel core-shell nanofiber drug delivery system intended for the synergistic treatment of melanoma. *European Journal of Pharmaceutical Sciences*. **2019**, 137, 105002.

[17] Zhu L-F, Yao Y, Ahmad Z, et al. Development of Ganoderma lucidum spore powder based proteoglycan and its application in hyperglycemic, antitumor and antioxidant function. *Process Biochemistry*. **2019**, 84, 103-111.
[18] Yoo KH, Park JH, Lee DY, et al. Corosolic Acid Exhibits Anti‐angiogenic and Anti‐lymphangiogenic Effects on In Vitro Endothelial Cells and on an In Vivo CT‐26 Colon Carcinoma Animal Model. *Phytotherapy Research*. **2015**, 29(5), 714-723.
[19] Ki Hyun Y, Jong-Hwa P, Dae Young L, et al. Corosolic Acid Exhibits Anti-angiogenic and Anti-lymphangiogenic Effects on In Vitro Endothelial Cells and on an In Vivo CT-26 Colon Carcinoma Animal Model. *Phytotherapy Research*. **2015**, 29(5), 714-723.
[20] Tiwari BK. Ultrasound: A clean, green extraction technology. *TrAC Trends in Analytical Chemistry*. **2015**, 71, 100-109.
[21] Vilkhu K, Mawson R, Simons L, et al. Applications and opportunities for ultrasound assisted extraction in the food industry — A review. *Innovative Food Science & Emerging Technologies*. **2008**, 9(2), 161-169.
[22] Hromádková Z, Ebringerová A, Valachovic P. Comparison of classical and ultrasound-assisted extraction of polysaccharides from Salvia officinalis L. *Ultrasonics Sonochemistry*. **1999**, 5(4), 163.
[23] Altemimi A, Lakhssassi N, Baharlouei A, et al. Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. *Plants*. **2017**, 6(4).
[24] Shirsath SR, Sonawane SH, Gogate PR. Intensification of extraction of natural products using ultrasonic irradiations—A review of current status. *Chemical Engineering & Processing Process Intensification*. **2010**, 49(3), 10-23.
[25] Soria AC, Villamiel M. Effect of ultrasound on the technological properties and bioactivity of food: a review. *Trends in Food Science & Technology*. **2010**, 21(7), 323-331.
[26] Chemat F, Rombaut N, Sicaire A-G, et al. Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrasonics Sonochemistry*. **2017**, 34, 540-560.
[27] Esclapez MD, García-Pérez JV, Mulet A, et al. Ultrasound-Assisted Extraction of Natural Products. *Food Engineering Reviews*. **2011**, 3(2), 108-120.
[28] Dranca F, Oroian M. Optimization of ultrasound-assisted extraction of total monomeric anthocyanin (TMA) and total phenolic content (TPC) from eggplant (Solanum melongena L.) peel. *Ultrasonics Sonochemistry*. **2016**, 31, 637-646.
[29] Xiang Z, Tang C, Chen G, et al. Studied on corlorimetric determination of oleanolic acid in Chinese quince. *Natural Product Research & Development*. **2001**, 13, 23-26.
[30] Floegel A, Kim D-O, Chung S-J, et al. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *Journal of Food Composition and Analysis*. **2011**, 24(7), 1043-1048.
[31] Huang Q, Chen L, Teng H, et al. Phenolic compounds ameliorate the glucose uptake in HepG2 cells' insulin resistance via activating AMPK: Anti-diabetic effect of phenolic compounds in HepG2 cells. *Journal of Functional Foods*. **2015**, 19, 487-494.
[32] Liu K, Liu P-c, Liu R, et al. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. *Medical science monitor basic research*. **2015**, 21, 15-20.

[33] Pinelo M, Rubilar M, Jerez M, et al. Effect of Solvent, Temperature, and Solvent-to-Solid Ratio on the Total Phenolic Content and Antiradical Activity of Extracts from Different Components of Grape Pomace. *Journal of Agricultural and Food Chemistry*. **2005**, 53(6), 2111-2117.

[34] Chen Y, Xie M-Y, Gong X-F. Microwave-assisted extraction used for the isolation of total triterpenoid saponins from Ganoderma atrum. *Journal of Food Engineering*. **2007**, 81(1), 162-170.

[35] Carciochi RA, Manrique GD, Dimitrov K. Optimization of antioxidant phenolic compounds extraction from quinoa (Chenopodium quinoa) seeds. *Journal of Food Science and Technology*. **2015**, 52(7), 4396-4404.

[36] Chavan Y, Singhal RS. Ultrasound-assisted extraction (UAE) of bioactives from arecanut (Areca catechu L.) and optimization study using response surface methodology. *Innovative Food Science & Emerging Technologies*. **2013**, 17, 106-113.

[37] Zhang Z-S, Li D, Wang L-J, et al. Optimization of ethanol–water extraction of lignans from flaxseed. *Separation and Purification Technology*. **2007**, 57(1), 17-24.

[38] Yang L, Cao Y-L, Jiang J-G, et al. Response surface optimization of ultrasound-assisted flavonoids extraction from the flower of Citrus aurantium L. var. amara Engl. *Journal of Separation Science*. **2010**, 33(9), 1349-1355.

[39] Hongzhang C, Liying L. Unpolluted fractionation of wheat straw by steam explosion and ethanol extraction. *Bioresource Technology*. **2007**, 98(3), 666-676.

[40] Mandal V, Mandal SC. Design and performance evaluation of a microwave based low carbon yielding extraction technique for naturally occurring bioactive triterpenoid: Oleanolic acid. *Biochemical Engineering Journal*. **2010**, 50(1), 63-70.

[41] Ying Z, Han X, Li J. Ultrasound-assisted extraction of polysaccharides from mulberry leaves. *Food Chemistry*. **2011**, 127(3), 1273-1279.

[42] Albero B, Tadeo JL, Pérez RA. Ultrasound-assisted extraction of organic contaminants. *TrAC Trends in Analytical Chemistry*. **2019**, 118, 739-750.

[43] Khadhraoui B, Turk M, Fabiano-Tixier AS, et al. Histo-cytochemistry and scanning electron microscopy for studying spatial and temporal extraction of metabolites induced by ultrasound. Towards chain detexturation mechanism. *Ultrasonics Sonochemistry*. **2018**, 42, 482-492.

[44] Knorr D, Ade-Omowaye BIO, Heinz V. Nutritional improvement of plant foods by non-thermal processing. *Proceedings of the Nutrition Society*. **2009**, 61(2), 311-318.

[45] Zhang Z-S, Wang L-J, Li D, et al. Ultrasound-assisted extraction of oil from flaxseed. *Separation and Purification Technology*. **2008**, 62(1), 192-198.

[46] Rodrigues S, Pinto GAS, Fernandes FAN. Optimization of ultrasound extraction of phenolic compounds from coconut (Cocos nucifera) shell powder by response surface methodology. *Ultrasonics Sonochemistry*. **2008**, 15(1), 95-100.

[47] Martínez-Tarifa A, Arrojo S, Louisnard O, et al. Correlation between hydroxyl radical production and theoretical pressure distribution in a sonochemical reactor. *Physics Procedia*. **2010**, 3(1), 971-979.

[48] Maran JP, Sivakumar V, Sridhar R, et al. Development of model for barrier and optical properties of tapioca starch based edible films. *Carbohydrate Polymers*. **2013**, 92(2), 1335-1347.
[49] Goldfarb HB. Experimental Design for Formulation. *Technometrics*. **2006**, 48(2), 304-304.
[50] Oludemi T, Barros L, Prieto MA, et al. Extraction of triterpenoids and phenolic compounds from Ganoderma lucidum: optimization study using the response surface methodology. *Food & Function*. **2018**, 9(1), 209-226.
[51] He R, Ye J, Zhao Y, et al. Partial characterization, antioxidant and antitumor activities of polysaccharides from Philomycusbilineatus. *International Journal of Biological Macromolecules*. **2014**, 65, 573-580.
[52] Altemimi A, Choudhary R, Watson DG, et al. Effects of ultrasonic treatments on the polyphenol and antioxidant content of spinach extracts. *Ultrasonics Sonochemistry*. **2015**, 24, 247-255.
[53] Ge Q, Mao JW, Guo XQ, et al. Composition and antioxidant activities of four polysaccharides extracted from Herba Lophatheri. *International Journal of Biological Macromolecules*. **2013**, 60, 437-441.
[54] Xiong Sl, Li A, Huang N, et al. Antioxidant and immunoregulatory activity of different polysaccharide fractions from tuber of Ophiopogon japonicus. *Carbohydrate Polymers*. **2011**, 86(3), 1273-1280.
[55] Jeong CH, Choi GN, Kim JH, et al. Antioxidant activities from the aerial parts of Platycodon grandiflorum. *Food Chemistry*. **2010**, 118(2), 278-282.
[56] Sh.Sh H, Huang GJ, Ho YL, et al. Antioxidant and antiproliferative activities of the four Hydrocotyle species from Taiwan. *Botanical Studies*. **2008**, 49(4), 311-322.
[57] Mathew S, Abraham TE. In vitro antioxidant activity and scavenging effects of Cinnamomum verum leaf extract assayed by different methodologies. *Food and Chemical Toxicology*. **2006**, 44(2), 198-206.
[58] DeFronzo RA, Hendler R, Simonson D. Insulin Resistance is a Prominent Feature of Insulin-dependent Diabetes. *Diabetes*. **1982**, 31(9), 795-801.

Tables and Figures

Table 1

Coded variable levels and outcomes for variables.

Variables		Coded levels of variables		
		-1	0	1
Ultrasound power (w)	A	450	525	600
Ultrasound time (min)	B	1	5.5	10
Ultrasound distance (cm)	C	4.5	8	11.5

Table 2

Experimental design and results for the Box-Behnken Model.

Test run no.	Coded levels of variables			Yield of triterpenoids (%)
	A	B	C	
1	1	1	0	0.82
2	1	-1	0	0.83
3	-1	1	0	1.02
4	-1	-1	0	1.04
5	1	0	1	0.78
6	1	0	-1	0.76
7	-1	0	1	0.97
8	-1	0	-1	0.96
9	0	-1	1	0.87
10	0	-1	-1	0.80
11	0	1	1	0.86
12	0	1	-1	0.81
13	0	0	0	1.05
14	0	0	0	1.06
15	0	0	0	1.06

Table 3

Variance analysis of *Ganoderma lucidum* triterpenoid yield.

Source	Sum of Squares	df	Mean Square	F Value	p-value	Significant
Model	0.18	9	0.02	79.84	< 0.0001	Significant
A-Ultrasound power	0.08	1	0.08	322.15	< 0.0001	
B-Ultrasound time	1.13E-04	1	1.13E-04	0.45	0.5308	
C-Ultrasound distance	2.81E-03	1	2.81E-03	11.33	0.02	
AB	2.50E-05	1	2.50E-05	0.1	0.7638	
AC	2.50E-05	1	2.50E-05	0.1	0.7638	
BC	1.00E-04	1	1.00E-04	0.4	0.5536	
A^2	8.63E-03	1	8.63E-03	34.73	0.002	
B^2	0.024	1	0.024	97.15	0.0002	
C^2	0.073	1	0.073	294.9	< 0.0001	
Residual	1.24E-03	5	2.48E-04			
Lack of Fit	1.18E-03	3	3.92E-04	11.75	0.0794	Not significant
Pure Error	6.67E-05	2	3.33E-05			
Cor Total	0.18	14				

Table 4
Variance analysis for fitted model.

	Model
Std. Dev.	0.016
Mean	0.91
C.V. %	1.73
PRESS	0.019
R-Squared	0.9931
Adj R-Squared	0.9807
Pred R-Squared	0.8945
Adeq Precision	24.125

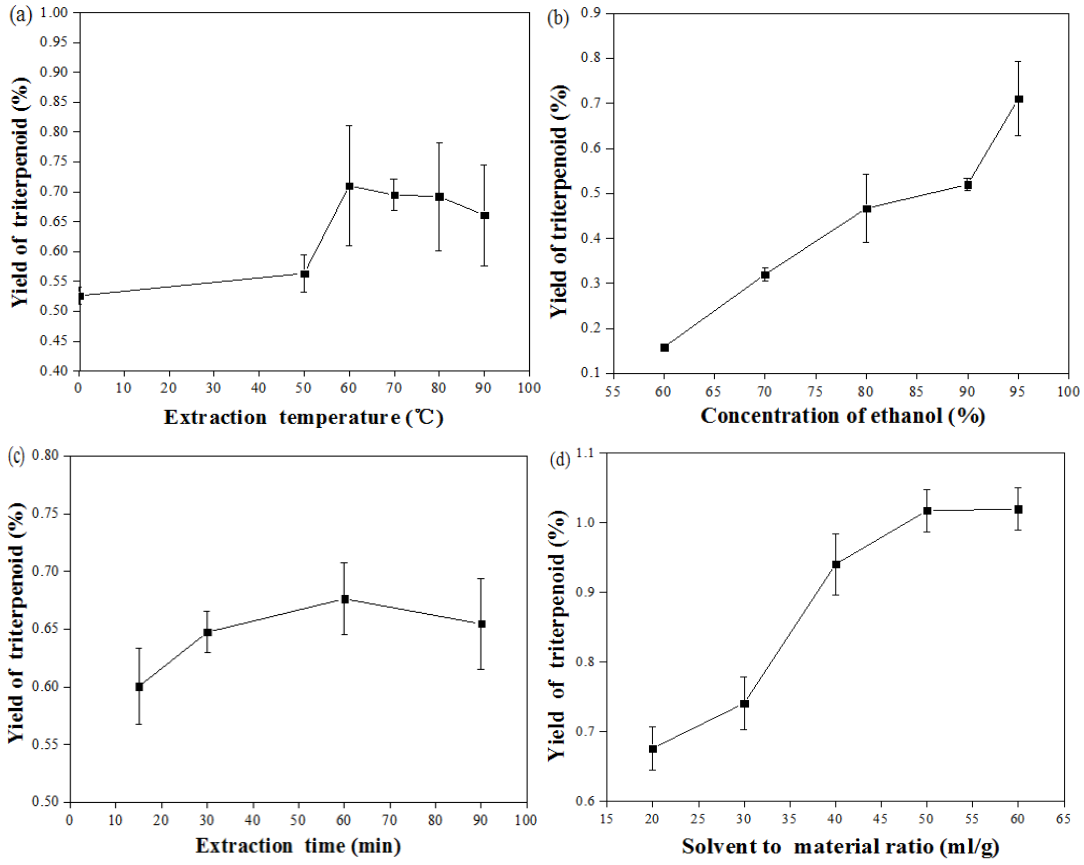


Figure.1. Effect of extraction variables on triterpenoid yield for 1 g GLSP. (a) Effect of extraction temperature with 20 mL of (95%) ethanol for 1 h; (b) Effect of ethanol concentration, 20 mL of solvent for 1 h at 60ºC; (c) Effect of extraction time with 20 mL (95%) ethanol at 60ºC; (d) Effect of solvent to material ratio (ml/g) with (95%) ethanol for 1 h at 60ºC. Each measurement was performed in triplicate. Each value represents a mean ± SD (n=3).

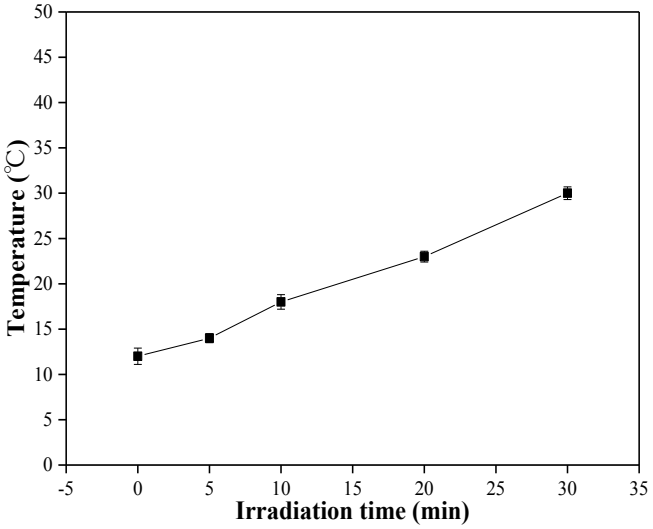


Figure.2. Relationship between temperature and irradiation time.

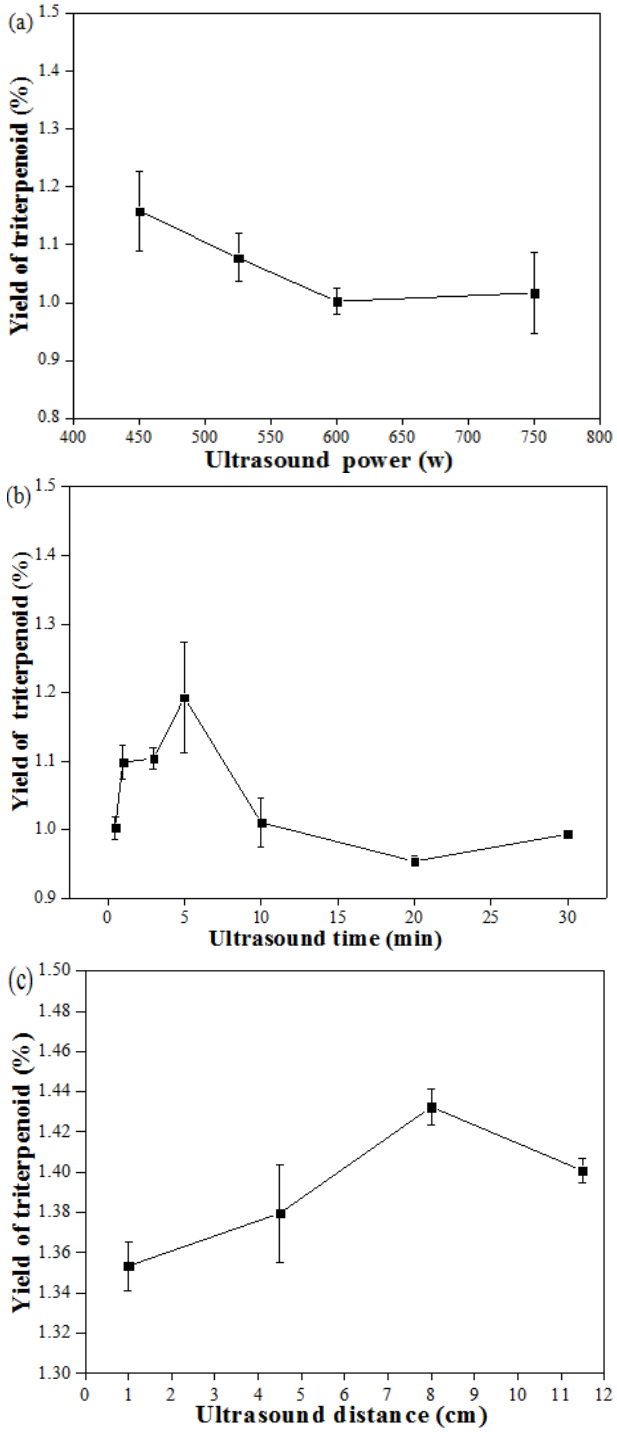


Figure.3. The effect of ultrasound extraction variables on triterpenoid yield. (a) Effect of ultrasound power, 1 g GLSP extracted with 50 mL (95%) ethanol for 10 min. (b) Effect of ultrasound time, 1 g GLSP extracted with 50 mL (95%) ethanol. Ultrasound power was 450 w. (c) Effect of ultrasound distance, 1 g GLSP extracted with 50 mL (95%) ethanol for 10 min. Ultrasound power=450 w. Each measurement was carried out in triplicate. Each value represents a mean ± SD (n=3).

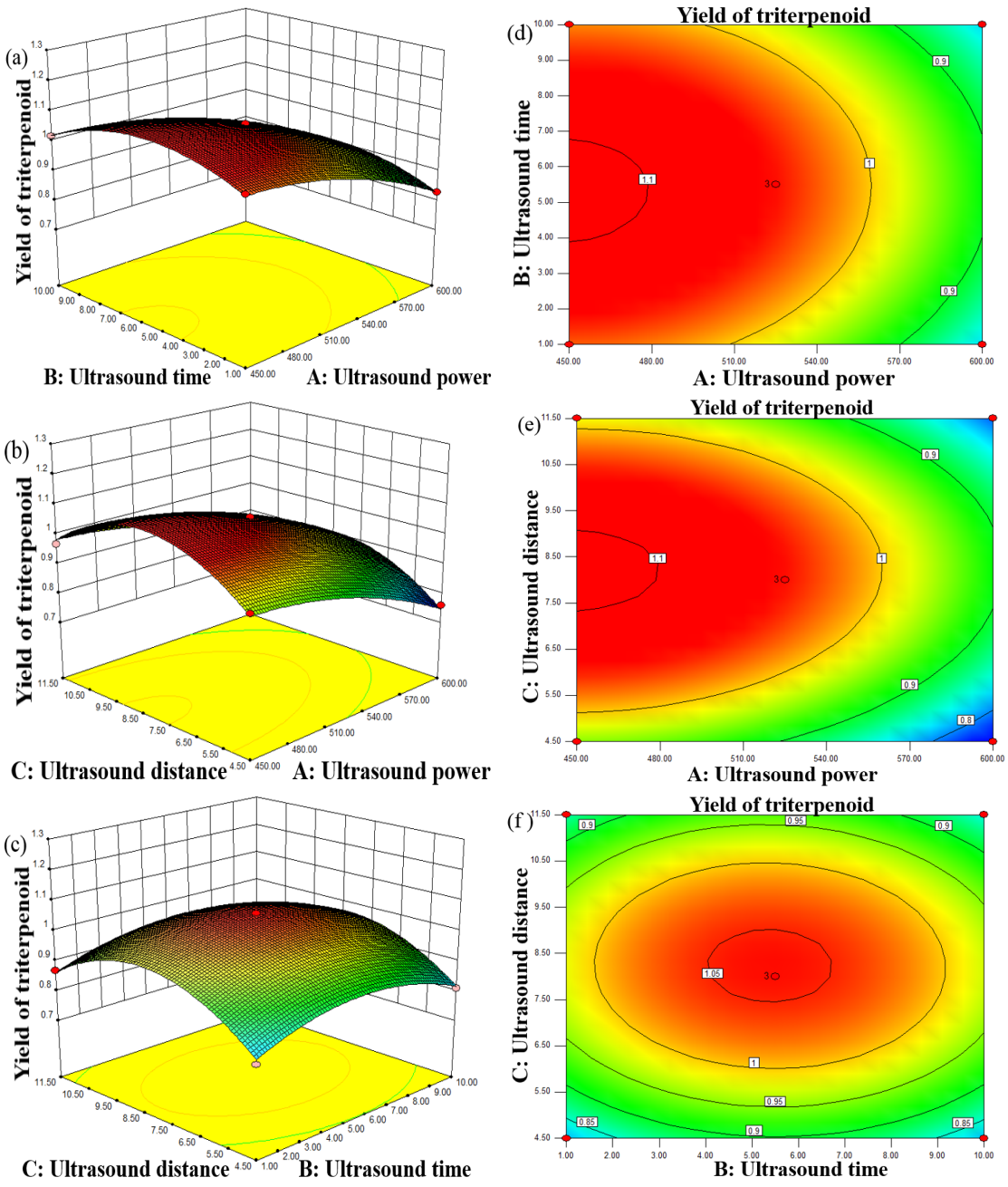


Figure.4. Three-dimensional and two-dimensional response surface contour plots showing the experiment factors and their mutual interactions on triterpenoids yield. (a) ultrasound power and ultrasound time; (b) ultrasound power and ultrasound distance; (c) ultrasound time and ultrasound distance; (d) ultrasound power and ultrasound time; (e) ultrasound power and ultrasound distance; (f) ultrasound time and ultrasound distance.

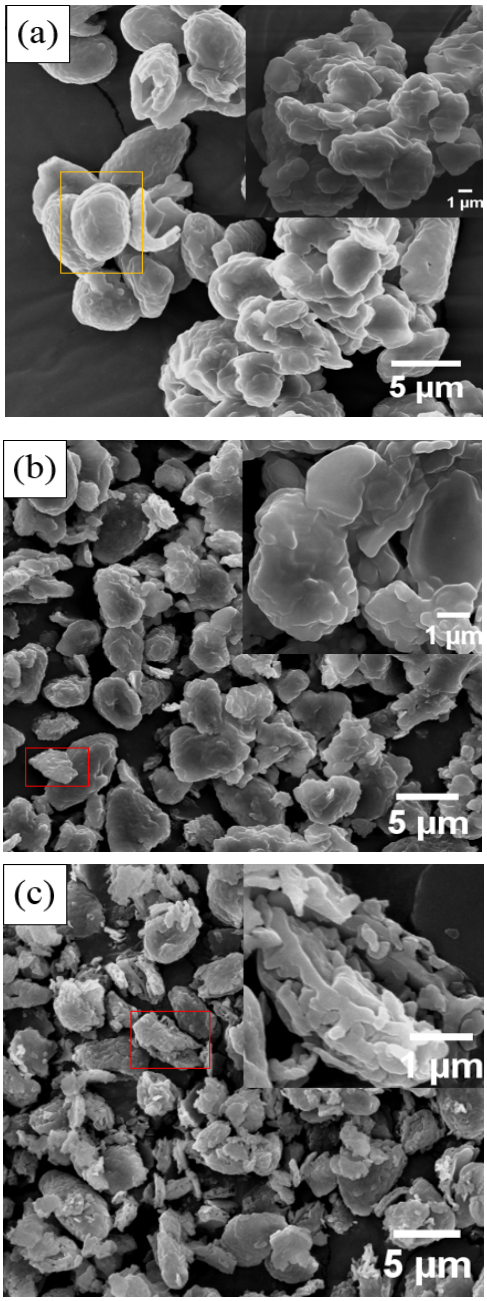


Figure. 5. Electron micrographs of various GLSP samples. (a) Untreated powder; (b) Post extraction using 95% ethanol at 60ºC for 1 h. (c) Post ultrasound assisted extraction using 450 w for 10 min. Insets in top right micrographs are high-magnification images. The yellow box shows normal GLSP while the red box shows damaged GLSP.

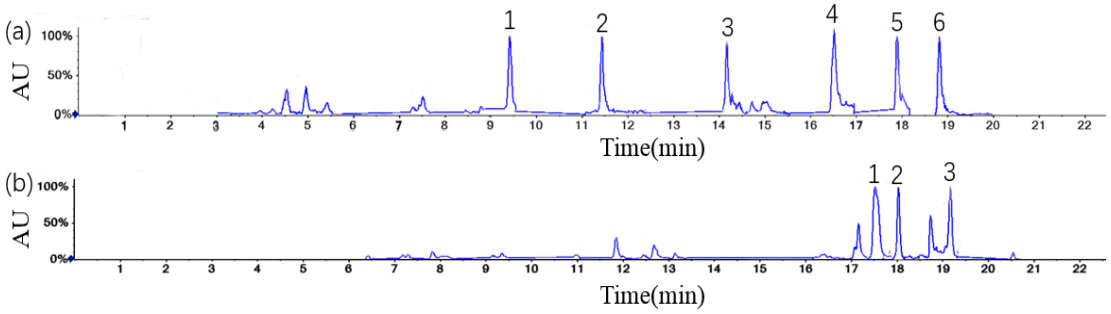


Figure. 6. Results of detection of triterpenoid extracts by fast high performance liquid-phase quadrupole time-of-flight (Q-TOF) mass spectrometry. (a) 1. ganoderic acid I 2. lucidenic acid D 3. ganoderenic acid D 4. ganodernoid C 5. ganoderic acid DM 6. ganodermic acid TQ. (b) 1. ganoderenic acid D 2. ganoderiol I 3. ganoderic acid C2.

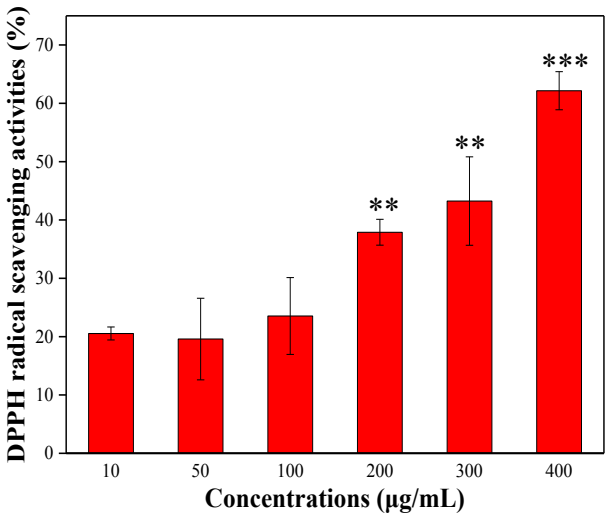


Figure. 7. DPPH radical-scavenging activities of triterpenoids from *Ganoderma lucidum* spore powder (GLSP). *p < 0.05, **p < 0.01, and ***p < 0.001 significantly different from 10 μg/mL.

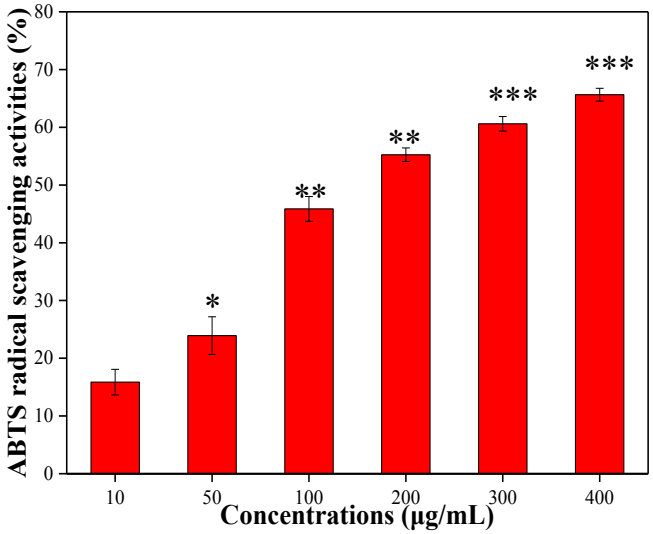


Figure. 8. ABTS radical-scavenging activity at varying concentrations of triterpenoids obtained from *Ganoderma lucidum* spore powder (GLSP). *p < 0.05, **p < 0.01, and ***p < 0.001 significantly different from 10 μg/mL.

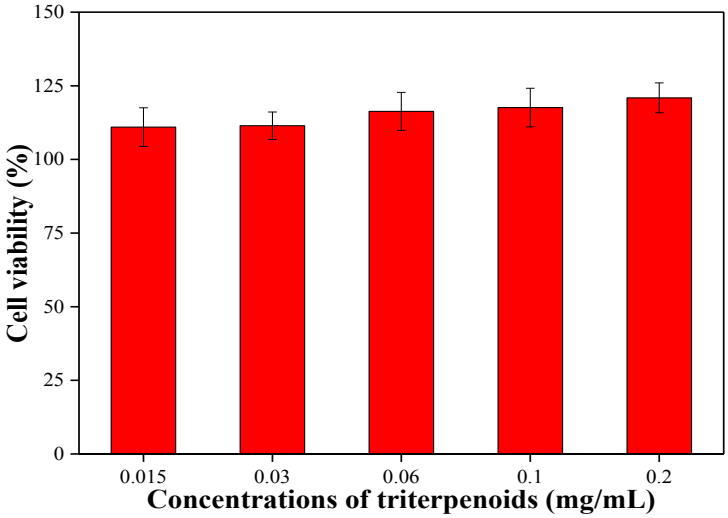
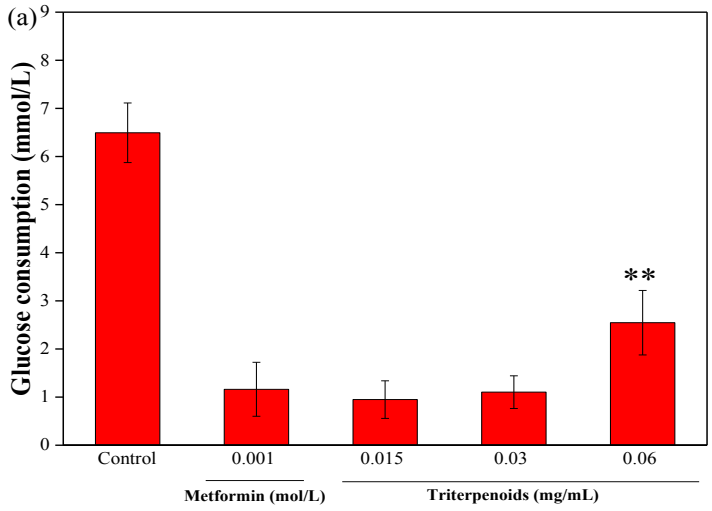


Figure. 9. HepG2 cell viability at varying triterpenoids concentrations (0.015, 0.03, 0.06, 0.1, 0.2 mg/mL).



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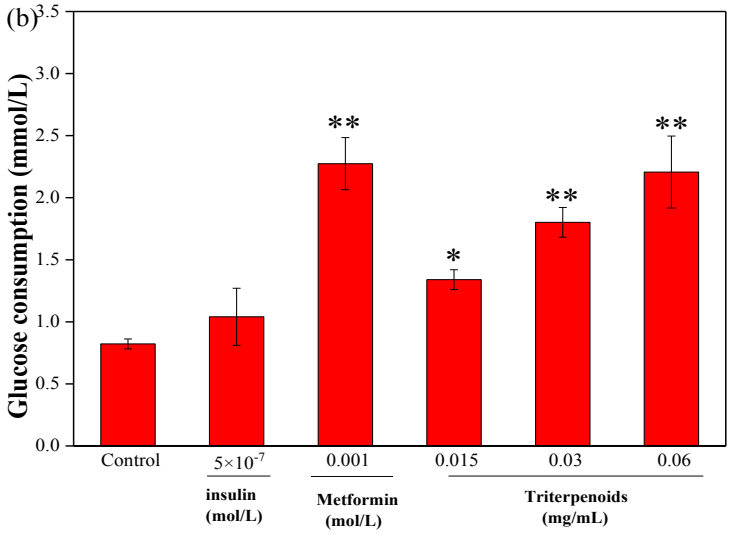

2

3 Figure. 10. (a) Effect of triterpenoids on normal HepG2 cell glucose consumption.
4 Metformin was positive control. (b) Effect of triterpenoids on insulin-resistant HepG2
5 cell glucose consumption. Both insulin and metformin were positive control. *p <
6 0.05, **p < 0.01, and ***p < 0.001 significantly different from blank control.

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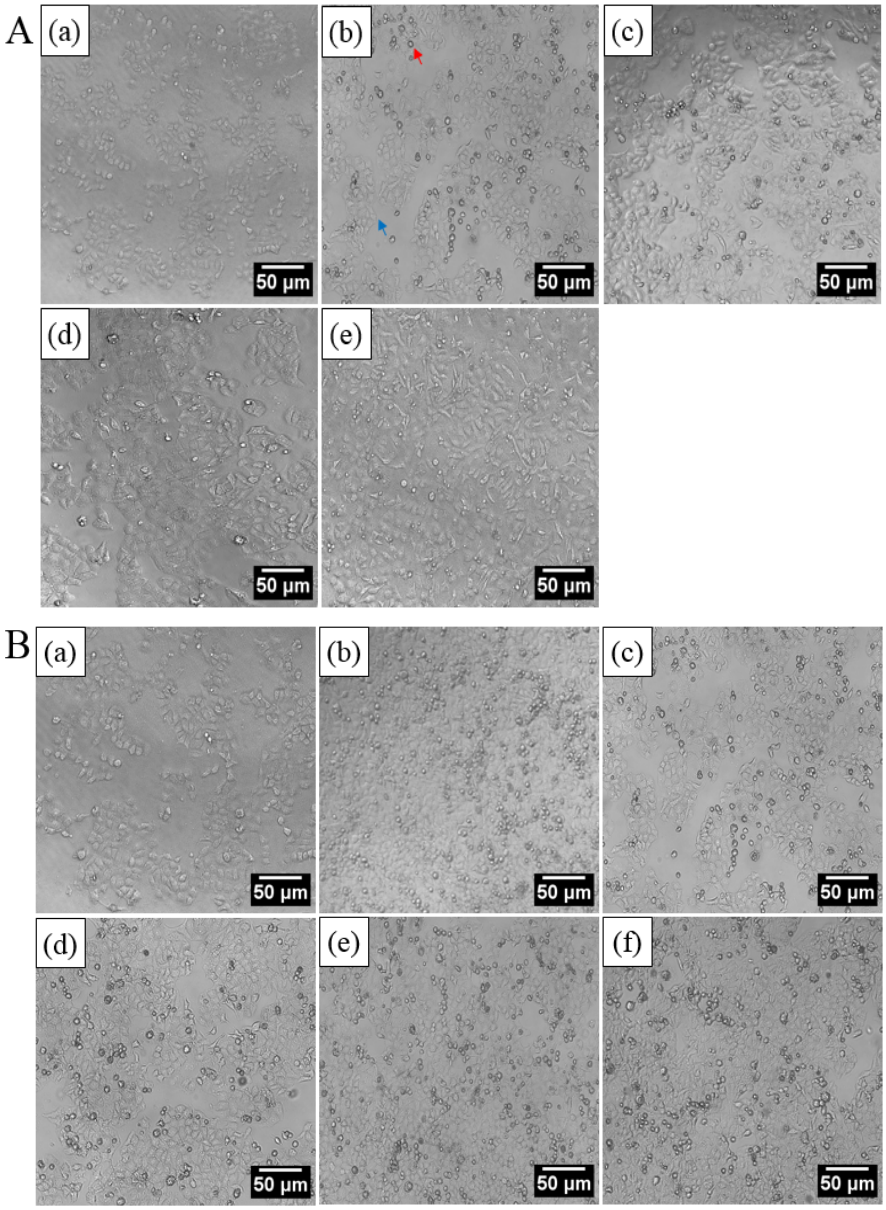

Figure. 11. Image of cell morphology taken by optical microscope. A. Effect of triterpenoids on normal HepG2 cell. (a) blank control; (b) metformin (0.001 mol/L); in the figure, the blue arrow represents normal cells and the red arrow represents damaged cells. (c) 0.015 mg/mL triterpenoids; (d) 0.03 mg/mL triterpenoids; (e) 0.06 mg/mL triterpenoids. B. Effect of triterpenoids on insulin-resistant HepG2 cell. (a) blank control; (b) insulin (5×10^{-7} mol/L); (c) metformin (0.001 mol/L); (d) 0.015 mg/mL triterpenoids; (e) 0.03 mg/mL triterpenoids; (f) 0.06 mg/mL triterpenoids.